Chromatographic separation of substances contained in a liquid sample.

Technical field

The present invention relates to a chromatographic separation method , a separation device and a process for the preparation of a separation medium for use therein. More particularly the present invention relates to a device and a method for the chromatographic separation of substances contained in a liquid sample and to a process for the preparation of a separation medium to be used in said method.

Background art

Chromatographic separation of substances contained in a liquid sample by applying said sample to a separation column in which a separation medium is arranged and eluting one or more product fractions from said column belongs to the well known prior art.

Traditionally chromatographic separation and isolation of substances has been done by sequential application of samples on one and the same chromatographic column. However, particularly in pharmaceutical research there is a large demand for the analysis of numerous samples. In order to facilitate the parallel chromatographic processing of a plurality of samples US-A-5 417 923, issued on May 23, 1995, suggests the use of an assay tray assembly comprising a one piece test tray mounted in over-lying relationship in engagement with a collection tray, which assay tray comprises a plurality of separation "columns" in the form of chambers in which a separation medium has been arranged. Figure 1 of US-A-5 417 923 shows an assay tray consisting of 96 chambers or columns arranged in 8 rows with 12 chambers in each row. The collection tray exhibits 96 wells arranged so as to enable direct trans-

fer of the chamber (column) effluents into the wells without intermixing or cross contamination.

The chromatographic media in the chamber is retained between underlying and overlying frits (items 31 and 32, respectively of figure 3) which usually are of a porous nature. Underlying frit 31 is retained on place by means of an annular flange 8 at the bottom end of each chamber thus providing a shoulder within the chamber 3. The liquid under test is usually pipetted into the columns in predetermined volumes. In liquid chromatography, the chromatographic column should always be filled with liquid, as the drainage of the column is detrimental for the chromatographic performance. To prevent uncontrolled column drainage, the liquid flow through the column is controlled either by applying some back pressure (which is released when the effluents are collected) or by using frits with a porosity which allows keeping the liquid inside the column due to the capillary forces. In the latter case the effluents are collected by creating artificial pressure drop above or below the column or by pushing liquid through the column due to the centrifugal forces.

C. Gottstein and R. Forde (Protein Engineering Vol. 15, No. 10, pp 775-777, 2002) disclose an affinity chromatography system for parallel purification of recombinant protein samples in which system a plurality of affinity columns (7) have been mounted in apertures in a common plate, liquid being provided to the columns by means of syringes (6), one for each column, and the flow through being collected in tubes (8). The columns (7) are filled with an affinity resin. This system allows the purification of 24 recombinant proteins in parallel.

It is an object of the present invention to provide a method for the chromatographic separation of substances contained in a liquid sample which method may be practised by using a system less complicated than those of the prior art disclosed above for the analysis of a plurality of samples in parallel.

It is another object of the present invention to provide a process for the preparation of separation media for use in a method for the chromatographic separation of substances for the analysis of a plurality of samples in parallel which separation medium enables the use of less complicated equipment than those disclosed by above cited prior art.

These and others objects are attained by means of the present invention.

Disclosure of the invention

According to one aspect of the present invention there is provided a method for the chromatographic separation of substances contained in a liquid sample comprising

providing a one piece separation tray having a spaced array of discrete identical upstanding chambers each exhibiting an open upper end and an open lower end and a separation medium placed in at least part of each upstanding chamber;

applying a liquid sample to said open upper end of at least one of said upstanding chambers;

then applying an eluting liquid to said open upper end of said at least one of said upstanding chambers; and

collecting at least one product fraction flowing out from the open lower end of said at least one of said upstanding chambers;

wherein a monolith of a compressible macroporous gel having in its liquid-swollen, non-compressed state a cross-sectional area which is 2-15 %, preferably 4-12 % and most preferably 5-10 %, larger than the cross-sectional area of the upstanding chamber in which it is placed is used as said separation medium and is in face-to-face contact with the wall of the respective chamber in its liquid-swollen state.

In accordance with one embodiment of this aspect of the present invention the method according to the present invention may be carried out using an assay tray assembly comprising a one piece test tray removably mounted in overlying relationship in engagement with a collection tray as disclosed by US-A-5 417 923, the disclosure of which is hereby incorporated herein by reference, the separation medium with underlying and overlying frits (31 and 32, respectively), however, being replaced by a rod-shaped monolith of a compressible macroporous gel as identified above.

By using as the separation medium, in accordance with the present invention, a monolith of a compressible macroporous gel which in its liquid-swollen, non-compressed state has a cross-sectional area which is somewhat larger, such as 2-15%, preferably 4-12% and most preferably 5-10% larger than the cross-sectional area of the lower part 6 of the chamber 3 of the prior art test tray, the annular flange 8 may be omitted since the monolith will be held retained on place due to the friction between the compressed gel monolith and the inner wall of the separation chamber. Thus the configuration of the assay tray is simplified. Moreover, there is no restriction in the flow area of liquid flowing out from the lower end of the separation chamber.

"A further advantage afforded by the separation medium used in the method according to the present invention is that the porous nature of the monolith enables liquid to be retained therein by capillary forces thus preventing the separation medium from "running dry."

US-A-5 417 923 disclosed each chamber to be formed with a conical top opening and a cylindrical lower part. In addition to such configuration the present invention contemplates the use of a chamber with a substantially constant cross-sectional area along its length. The configuration of the chambers as having an unaltered cross-sectional area along its length considerably simplifies the manufacture of the

separation tray. Moreover, although a circular cross-section of each chamber is the preferred one, any arbitrary cross-sectional configuration may be contemplated according to the present invention provided that the cross-section of the gel monolith is adapted thereto in order to obtain face-to-face contact in liquid-swollen state with the wall of the chamber within which it is placed. Thus the cross-sections of the chamber and the monolith may, for instance, form a triangle, a square, a rectangle, an oval, a pentagon, a hexagon, and so on, and may even exhibit an irregular shape.

Although US-A-5 417 923 discloses the separation media to occupy only part of the lower part of the chamber the present invention in addition contemplates the monolith to occupy fully the lower cylindrical (or other cross-sectional shape) part of the chamber or even to occupy fully the whole chamber in case of a chamber having a constant cross-sectional area along its length.

In order to avoid the necessity of applying supporting means, such as a net, for instance, at the open lower end of the upstanding chambers the monolith should exhibit a ratio between the length thereof in the longitudinal direction of the chamber and its cross-sectional dimensions exceeding a critical value below which the monolith may be bent so that the face-to-face contact with the chamber wall is lost. Thus, for instance, in case of cylindrical monoliths and chambers the length of the monolith should preferably exceed 50%, more preferably 60%, and most preferably 70% of the diameter of the monolith.

Monoliths of compressible macroporous gels to be used in the method according to the present invention can be prepared from various polymerisation systems.

Thus, according to one preferred embodiment of the method according to the present invention the monolith of a compressible macroporous gel is a cryogel that has been obtained by

polymerizing a solution of one or more monomers selected from the group consisting of:

N-substituted and non-substituted (meth)acrylamides;
N-alkyl substituted N-vinylamides;
hydroxyalkyl (meth)acrylates;
vinylacetate;
alkylethers of vinyl alcohol;
styrene and ring-substituted styrene derivatives;
vinyl monomers;
(meth)acrylic acid and salts thereof;
silicic acid; and
monomers capable of forming polymers via polycondensation

under freezing at a temperature below the solvent crystallization point, at which solvent in the system is partially frozen with the dissolved substances concentrated in the non-frozen fraction of solvent to the formation of a cryogel.

Details on such cryogels and the preparation thereof are found in International Patent Application No. PCT/SE02/01856 (WO 03/), the disclosure of which is hereby incorporated herein in its entirety by reference.

The solvent or solvent system used when preparing the cryogels may be selected from the group consisting of water, water-miscible organic solvents, mixtures of water and water-miscible organic solvents and mixtures of organic solvents but preferably water or a mixture of water and a water miscible organic solvent is used as solvent in combination with water-soluble monomers of the group recited above.

According to another preferred embodiment of the method according to the present invention the monolith of a compressible macroporous gel is a cryogel that has been obtained by cooling an aqueous solution of polyvinyl alcohol or at least one gel-forming polysaccharide selected from the group consisting of agarose, agar, carrageenans, starch and cellulose and their respective derivatives or a mixture of said poly-

saccharides to a temperature, at which the solvent in the system is partially frozen with the dissolved substances concentrated in the non-frozen fraction of the solvent to the formation of a cryogel, said cooling being carried out, when necessary, in the presence of at least one chaotropic agent in said aqueous solution in order to prevent gel formation before the polymer solution is frozen.

Details on such cryogels and the preparation thereof are found in the International Patent Application No. PCT/SE02/01857 (WO 03/) the disclosure of which is hereby incorporated herein in its entirety by reference.

In addition to the preparation of the monolith by moulding a monolith to be used in the method of the present invention may be formed by rolling or folding a sheet of a cryogel.

Moreover, in the method according to the present invention a monolith to be used therein may have been produced by a method selected from the group consisting of gel formation in double emulsion systems; freeze-drying of a polymer solution; leaching of a particulate material used as a porogen from a preformed polymer monolith; use of gas bubbles as a porogen when gel formation proceeds in foam; and aggregation of polymer particles or fibres (non-woven materials).

In addition to the methods for the preparation of macroporous gels recited above other existing methods or methods disclosed in the future resulting in macroporous compressible gels may in principle be used for the preparation of monoliths for use in the method according to the present invention.

The macroporous cryogels prepared by using the systems according to the preferred embodiments mentioned above may be modified in different ways as disclosed in PCT/SE02/01856 (WO 03/) and PCT/SE02/01857 (WO 03/).

Thus, for instance, the cryogels prepared from monomers as well as the cryogels based on polysaccharides or polyvinyl alcohol may have become cross-linked, e.g. by means of a cross-linking agent selected from the group consisting of alkylene diamines, glutaric aldehyde, di- and triglycidyl compounds and divinylsulfone in the first case and epichlorohydrin, divinyl sulfone, glutaric dialdehyde, azidobenzoyl hydrazide, 4-(N-maleimidomethyl)cyclohexane-1-carboxyl hydrazide hydrochloride, N-hydroxy-succinimidyl-4-azidosalicylic acid, 3-(2-pyridyl-dithio)-propinoyl hydrazide, dimethyladipimidate- 2HCl, N-succinimidyl-6-(4'-azido-2'-nitro-phenylamino)hexanoate and sulfosuccinimidyl-(4'-azidosalicylamido)hexanoate, di- and triglycidyl compounds in the second case.

Alternatively, or in addition thereto, the cryogel may have become modified by introducing a member selected from the group consisting of ligands, charged groups and hydrophobic groups thereinto.

According to another aspect of the present invention there is provided a separation device for use in a method according to the invention for the chromatographic separation of substances contained in a liquid sample, which separation device comprises a one piece separation tray having a spaced array of discrete identical upstanding chambers each exhibiting an open upper end and an open lower end and a separation medium placed in at least part of each upstanding chamber wherein said separation medium comprises a monolith of a compressible macroporous gel having in its liquid-swollen, non-compressed state a cross-sectional area which is 2-15 %, preferably 4-12 % and most preferably 5-10 %, larger than the cross-sectional area of the upstanding chamber in which it is placed, which monolith is in face-to-face contact with the wall of the respective chamber in its liquid-swollen state.

In its simplest embodiment the one piece separation tray comprises a plate of a rigid, inert material, such as a metal or

a polymer, provided with a plurality of cylindrical bores forming the upstanding chambers therein housing the separation media.

Other possible embodiments of the one piece separation tray should be evident from the description of the method of the invention above.

Detailed description of monoliths to be used in the separation device according to the invention is found above in connection with the method of the invention and below in connection with the process for the preparation of a separation medium for use in the method of the present invention.

According to further aspect of the present invention there is provided a process for the preparation of a separation medium for use in a method according to the present invention, which process comprises

a) providing a mould of a rigid material having a plurality of apertures going therethrough, said apertures having a cross-sectional configuration adapted to that of the upstanding chambers of a separation tray in which the separation medium prepared is to be used but having a cross-sectional area which is 2-15%, preferably 4-12% and most preferably 5-10% larger than that of said upstanding chambers;

b) sealing the apertures of the mould at the bottom thereof by means of an impervious removable plate;
c) introducing a solution of substances from which cryogels may be prepared into the apertures of the mould;
d) cooling the mould with said solution within the apertures thereof at a temperature below the solvent crystallisation point at which solvent in the system is partially frozen with the dissolved substances concentrated in the non-frozen fraction of solvent to the formation of a cryogel monolith;

e) defrosting the mould with the cryogel monoliths contained therein;

f) replacing the impervious removable plate by net with openings large enough to allow free flow of liquids therethrough but small enough to prevent the cryogel monoliths formed from passing therethrough;

g) washing the monoliths using a suitable washing medium; and

h) removing the monoliths from the mould.

In accordance with an alternative of this method steps e) and f) may be carried out in reverse order.

The phrase "said apertures having a cross-sectional configuration adapted to that of the upstanding chambers" as used here and in the claims should mean, for instance, that in the case when the upstanding chambers are cylindrical in shape the apertures of the mould are also cylindrical but having somewhat wider diameter in order to attain a cross-sectional area which is somewhat larger than that of the upstanding chambers. Similarly, in case of a triangular cross-section of the upstanding chambers, the apertures of the mould would also be of triangular cross-section with angles identical to those of the upstanding chambers but having somewhat longer sides.

There is no specific restriction made on the cross-sectional configuration of the apertures of the mould and the upstanding chambers of the separation tray, as indicated previously. However, a circular cross-section is preferred from practical point of view.

The solution to be used in step c) of the process according to the present invention may be any solution previously used in the manufacture of cryogels.

In accordance with one preferred embodiment of the process according to the present invention said solution used in step

c) is a solution of one or more monomers selected from the group consisting of:

N-substituted and non-substituted (meth)acrylamides;
N-alkyl substituted N-vinylamides;
hydroxyalkyl (meth)acrylates;
vinylacetate;
alkylethers of vinyl alcohol;
styrene and ring-substituted styrene derivatives;
vinyl monomers;
(meth)acrylic acid and salts thereof;
silicic acid; and
monomers capable of forming polymers via polycondensation

under freezing at a temperature below the solvent crystallization point, at which solvent in the system is partially frozen with the dissolved substances concentrated in the non-frozen fraction of solvent to the formation of a cryogel.

Details on such cryogel and the preparation thereof are found in International Patent Application No. PCT/SE02/01856 (WO 03/), the disclosure of which is hereby incorporated herein in its entirety by reference.

The solvent or solvent system used when preparing the cryogels may be selected from the group consisting of water, water-miscible organic solvents, mixtures of water and water-miscible organic solvents and mixtures of organic solvents but preferably water or a mixture of water and a watermiscible organic solvent is used as solvent in combination with water-soluble monomers of the group recited above.

In accordance with another preferred embodiment of the process according to the present invention said solution used in step c) is an aqueous solution of polyvinyl alcohol or at least one gel forming polysaccharide obtained by cooling an aqueous solution of polyvinyl alcohol or at least one gel forming polysaccharide selected from the group consisting of agarose, agar, carrageenans, starch and cellulose and their

respective derivatives or a mixture of said polysaccharides to a temperature, at which the solvent in the system is partially frozen with the dissolved substances concentrated in the non-frozen fraction of solvent to the formation of a cryogel said cooling being carried out, when necessary, in the presence of at least one chaotropic agent in said aqueous solution in order to prevent gel formation before the polymer solution is frozen.

Details on such cryogels and the preparation thereof are found in the International Patent Application No. PCT/SE02/01857 (WO 03/) the disclosure of which is hereby incorporated herein in its entirety by reference.

According to a modification of the process according to the present invention the cryogel monoliths formed may be subjected to one or more chemical modifications before step h), e.g. as disclosed in PCT/SE02/01856 (WO 03/) and PCT/SE02/01857 (WO 03/).

Thus, for instance, the cryogels prepared from monomers as well as the cryogels based on polysaccharides or polyvinyl alcohol may be subjected to a cross-linking reaction, e.g. by reaction with a cross-linking agent selected from the group consisting of alkylene diamines, glutaric aldehyde, di- and triglycidyl compounds and divinylsulfone in the first case and epichlorohydrin, divinyl sulfone, glutaric dialdehyde, azidobenzoyl hydrazide, 4-(N-maleimidomethyl)cy-clohexane-1-carboxyl hydrazide hydrochloride, N-hydroxy-succinimidyl-4-azidosalicylic acid, 3-(2-pyridyl-dithio)-propinoyl hydrazide, dimethyladipimidate- 2HCl, N-succini-midyl-6-(4'-azido-2'-nitro-phenylamino)hexanoate and sulfosuccinimidyl-(4'-azidosalicylamido)hexanoate, di- and triglycidyl compounds in the second case.

Alternatively, or in addition thereto, the cryogel monoliths may be modified by introducing a member selected from the

group consisting of ligands, charged groups and hydrophobic groups thereinto.

Examples of ligands which may be introduced into the cryogel monoliths prepared according to the present invention are peptides, metal chelates, sugar derivatives, boronate derivatives, enzyme substrates and their analogues, enzyme inhibitors and their analogues, protein inhibitors, antibodies and fragments thereof, ligand structures obtained via combinatorial chemistry and thiol-containing substances.

Examples of charged groups which may be introduced into the cryogel monoliths prepared according to the present invention are ion exchange groups.

According to the present invention there is also provided an alternative process for the preparation of a separation medium for use in a method according to the invention, which process comprises

a) providing an elongated, tubular mould having a closed end and having a cross-sectional configuration adapted to that of the upstanding chambers of a separation tray in which the separation medium prepared is to be used but having a cross-sectional area which is 2-15%, preferably 4-12% and most preferably 5-10% larger than that of said upstanding chambers;

b) introducing a solution of substances from which cryogels may be prepared into the mould;

c) cooling the mould with said solution in it at a temperature below the solvent crystallisation point at which solvent in the system is partially frozen with the dissolved substances concentrated in the non-frozen fraction of solvent to the formation of a cryogel monolith;

d) defrosting the mould with the cryogel monolith contained therein;

e) removing the closed end of the mould;

f) washing the monolith by passing a suitable washing medium through the mould;

g) removing the monolith from the mould; and

h) cutting the monolith into pieces of a size suitable for use in an upstanding chamber of a separation tray.

Solutions to be used in this alternative process may be those already mentioned in connection with the previously discussed process and modifications to the cryogels prepared may also be performed as discussed above.

According to the present invention there is also provided a further alternative process for the preparation of a separation medium for use in the method according to the invention, which process comprises

a) extruding a solution of substances from which cryogels may be prepared directly into a cold organic medium which is a non-solvent for the solutes of said solution to the formation of a continuous string of substantially uniform cross-section in which gel formation takes place; and

b) cutting the string into pieces of a size suitable for use in an upstanding chamber of a separation tray.

Cryogel monoliths prepared according to the processes according to the present invention may be inserted at least one into each upstanding chamber of a separation tray either in liquid swollen or dried state. When inserting dried monoliths the transient use of a removable net or film under the separation tray may advantageously be applied to keep the monoliths in place. The net or film is removed and the cryogels are soaked inside the upstanding chambers before the use of the separation tray.

The method according to the present invention may, as indicated previously, be practiced using an assay tray assembly as disclosed by US-A-5 417 923 and preferably with the test tray modified as indicated above. The collection tray may also be modified in comparison with that disclosed in US-A-5 417 923 without departing from the scope of the present invention as defined in the claims. Thus, for instance, a conventional microtiter plate having wells in a number and a mutual relationship (locations) corresponding to the upstanding chambers of the separation tray may be used as the collection tray. The number of upstanding chambers of the separation tray and of the wells of the collection tray may be one of those conventionally used in microtiter plates, such as, for instance, 48, 96, 192 or 384 wells.

The invention will now be illustrated by means of a number of working examples which are for illustrative purpose only and not intend as limitations.

Example 1. Production of cryogel monoliths and separation tray

Dimethylacrylamide (DMAA; 1.85 ml purified from stabilisator on an aluminium oxide column) and N,N'-methylene-bis(acrylamide) (MBAAm; 0.663 g) were dissolved in 40 ml of deionized water. Then 0.243 ml of allyl glycidyl ester (AGE) was added to the mixture at continuous stirring and the volume was adjusted to 50 ml with deionized water. The mixture was degassed under vacuum for 10-15 min to eliminate dissolved oxygen. Free radical polymerization was initiated by N,N,N',N'-tetra-methyl-ethylenediamine (TEMED) and ammonium persulfate (APS). After addition of TEMED (31.25 µl) the solution was cooled in an ice bath for 15 min. A freshly prepared solution of APS was added (final amount of APS in the reaction mixture 26.3 mg) and the mixture was stirred gently for 1 min. Then 0.5 ml of the reaction mixture was added very quickly into each well (diameter 7.1 mm) of a metal microtiter plate mold with 96 wells (the pipette tips used for the

filling of the mold with a multichannel pipette and the mold was cooled at -12° C prior to the filling). The metal plate has 96 cylindrical holes having a diameter of 7,1 mm drilled all through the material. The plate was placed on a metal plate making the bottom tight so that when filling in the solution it stays at the bottom of the hole, where the gel is formed. The solution in the microtiter plate mold was frozen within 10 min in the ARCTEST cooling chamber at -12° C. The samples were kept frozen at -12° C over night and then thawed at room temperature. The metal plate sealing the mould at the bottom thereof was replaced by a wire net. The cryogel matrix was then washed by passing 10 ml of deionized water through each well. Then the cryogels were transferred from the metal 96-well mold into a plastic microtiter plate developed for chromatography applications (microtiter plate with 96 bottomless round wells, well volume of 1.5 ml, well diameter 6.9 mm). After filling with the monolith each well has about 1 ml dead volume above the monolith. The microtiter plate with the monoliths contained in the wells thereof constitutes a separation tray for use in a method according to the invention

Example 2. Coupling of Cu2+ IDA ligand to the cryogels monoliths in the separation tray,

Four ml of 0.5 M Na2CO3 solution were passed through each well packed with cryogel monolith (0.5 ml) of a separation tray as disclosed in Example 1. Then each well was equilibrated with 0.5 M IDA in 1.0 M Na2CO3, pH 10.0. Finally, the microplate with the cryogel monoliths was placed into a vessel containing 400 ml of 0.5 M IDA in 1.0 M Na2CO3, pH 10.0 and was incubated overnight at room temperature and gentle shaking. The modified cryogels in the microplate were washed with water until pH became neutral. Cu2+ was bound to the IDA-cryogels by passing 2 ml of 0.5 M CuSO4 (dissolved in distilled water) through each well. Finally, each well was washed with water and equilibrated with 20 mM HEPES, 0.2 M NaCl pH 7.0.

Example 3. Chromatography of lysozyme on Cu2+-IDA cryogel separation tray

Different amounts of lysozyme (25, 50, 75, 100, 125, 150, 175, 200 and 250 µg) dissolved in 0,5 ml 20 mM HEPES, 0,2 M NaCl, pH 7,0, were applied to Cu2+ IDA cryogel monoliths in the different wells of the separation tray prepared as disclosed in Example 2. The monoliths were preequilibrated with 20 mM HEPES, 0.2 M NaCl pH 7.0 (running buffer). After the adsorption stage each analyzed well was washed with 2 ml of the running buffer. Bound lysozyme was eluted with 2.5 ml of 0.2 M imidazole in the running buffer. The chromatography with each lysozyme concentration was performed on 5 randomly selected wells. (Table 1). The average deviation between different wells was 6-8 %.

Table 1. The dependence of the amount of recovered lysozyme on the amount of lysozyme applied to Cu2+ IDA cryogel monoliths

Applied lysozyme, µg	0	50	75	100	125	150	175	200	250
Bound lysozyme, µg	0	10	28	40	55	80	107	115	116

The kinetics of lysozyme recovery is presented in Table 2 when 25 µg lysozyme was applied on randomly selected wells.

Table 2. The kinetics lysozyme on the amount of lysozyme recovery from Cu2+ IDA cryogel monoliths

Time, min	0	10	20	40	80
Recovered Lysozyme, %	72	80	92	92	96

Example 4. Direct quantification of lactate dehydrogenase from crude homogenate using monolith separation trays

Different amounts of E. coli cell homogenate containing histidine (His6)-tagged lactate dehydrogenase (LDH) were applied to the Cu2+ IDA cryogel monoliths in the different wells (0.5 ml) of a separation tray according to Example 2 equilibrated with 0.2 M TRIS buffer pH 7.3 (buffer A). After 5 min incubation the wells were washed with the same buffer to remove unbound protein and cell debris. Then the reaction mixture (0.5 ml buffer A containing 0.45 mM NADH and 2.0 mM pyruvate) was added and incubated within the wells for 2 min. After incubation the reaction mixture was "pushed out" from the wells by adding 0.5 ml of buffer A and analysed by measuring absorbance at 340 nm. In the control experiment buffer A was used instead of cell homogenate. ΔA340 is the difference between the obtained values of absorbance at 340 nm in the reaction mixtures from control and homogenate-loaded wells. Each homogenate load was analysed on 5 different randomly chosen wells. ΔA340 increased with increasing amount of homogenate applied allowing direct estimation of the amount of LDH present in the sample. Average deviation of the experiment was 7.5 %.

Example 5. Production of monolithic cryogel from polyvinyl alcohol (monolithic cryoPVA) and separation tray

Aqueous solution (5 % w/v) of polyvinyl alcohol (PVA, molecular weight 67 000, degree of hydrolysis 87.7 %) was prepared and pH was adjusted to 1.0 with 5 M HCl. The solution was cooled in an ice bath for 10 min. The cross-linker glutaraldehyde (0.5 % w/v) was added and the reaction mixture was stirred for 1 min. Then 0.5 ml of the solution was added very quickly into each well (diameter 7.1 mm) of a metal microtiter plate mold with 96 wells (the pipette tips used for the filling of the mold with a multichannel pipette and the mold were cooled at -15° C prior to the filling). The metal plate has 96 cylindrical holes having a diameter of 7,1 mm drilled

all through the material. The plate is connected to a metal plate making the bottom tight so that when filling in the solution it stays at the bottom of the hole, where the gel is formed. The solution in the microtiter plate mold was frozen within 10 min in the ARCTEST cooling chamber at -15 °C. The samples were kept frozen at -15 °C over night and then thawed at room temperature. The metal plate sealing the mould at the bottom thereof was replaced by a wire net. The cryogel matrix was then washed by passing 5 ml of deionized water through each well. Then the cryogels were transferred from the metal 96-well mold into a plastic microtiter plate developed for chromatography applications (microtiter plate with 96 bottomless round wells, well volume of 1.5 ml, well diameter 6.9 mm). After filling with the monolith each well has about 1 ml dead volume above the monolith. The microtiter plate with the monoliths contained in the wells thereof constitutes a separation tray for use in a method according to the invention.

Example 6. Coupling of functional epoxy-groups to the monolithic cryoPVA in a separation tray

A separation tray with monolithic cryoPVA was prepared according to Example 5. Deionized water (5 ml) followed by 5 ml of 0.1 M NaOH were passed through each well. An epichlorohydrin emulsion in 0.5 M NaOH (2 %, v/v) was applied to the column in recycle mode for 48 hr. Finally, wells packed with epoxy-containing monolithic cryoPVA were washed with deionized water until pH became less than 7.

Example 7. Production of ion-exchange monolithic cryoPVA in a separation tray

A separation tray with epoxy-containing monolithic cryoPVA was prepared according to Example 6. Deionized water (5ml) followed by 5 ml of 0.1 M Na-carbonate buffer, pH 9.5 were passed through each well packed with epoxy-containing monolithic cryoPVA (0.5 ml). A solution of 3-dimethylaminopropylamine in 0.1 M Na-carbonate buffer, pH 9.5 was applied

in recycle mode for 24 hr. Finally, wells packed with ion-exchange monolithic cryoPVA were washed with deionized water until pH became less than 7.

Example 8. Production of rolled cryogel from acrylamide

Acrylamide (AAm, 3.76 g) and 1.62 g of N,N'-methylene-bis(acrylamide) (MBAAm) were dissolved in 80 ml water. The total volume was adjusted to 100 ml with deionized water. The mixture was degassed under vacuum for 10-12 min to eliminate dissolved oxygen. Free radical polymerization was initiated by N,N,N',N'-tetra-methyl-ethylenediamine (TEMED) and ammonium persulfate (APS). After addition of TEMED (63 μl) the solution was cooled in an ice bath for 20 min. A freshly prepared solution of APS (48 mg in 0.5 ml water) was added and the mixture was stirred for 1 min. Then the reaction mixture was poured in metallic round shape mould (height of the mould 1 cm and diameter 10 cm) on half of the mould height (i.e. 0.5 cm). The surface of the reaction mixture was covered with light plastic lid. The mixture was frozen at -18oC for about 16-19 min. The frozen sample was stored at -18oC overnight. After thawing the cryogel sheet was washed with deionized water. The round shaped cryogel sheet was put on a smooth surface and a rectangular 8 cm x 7 cm was cut. Then the free water was squeezed out lightly from the square shaped cryogel sheet. The cryogel sheet was rolled and placed into a glass tube with diameter 1.9 cm. The height of the cryogel column was 8 cm and the volume was 20 ml (1.4 times less then the total volume of the cryogel sheet, 28 ml). Compressing of the cryogel column diameter was important to avoid any space in rolled sheet, especially in the middle of the rolled sheet. The glass column was fitted with adapters and was connected to a pump. Na2CO3 (0.5 M, 200 ml) was passed through the column at a flow rate of 1 ml/min. The solution of iminodiacetic acid (0.5 M in 1.0 M Na2CO3) was applied to the column at a flow rate of 2 ml/min in recycle mode for 24 hr. After that the column was washed with deionized water (until pH became neutral). Cu2+ was bound to the IDA-cryogel column by passing 100 ml of 0.2 M CuSO4 through the column

100 ml of 0.2 M CuSO4 through the column followed by washing with water.

Example 9. Immobilization of concanavalin A on epoxy-activated cryogel monoliths in a separation tray

Ten epoxy-activated cryogel monoliths (0.5 ml) were placed into a separation tray prepared according to Example 6. Two ml of 0.05 M carbonate buffer pH 9.0 containing 1 M NaCl, 1 mM CaCl2, 1 mM MgCl2 (buffer A) were passed through each monolith followed by application of 0.5 ml of concanavalin A (ConA) solution (4 mg/ml) in buffer A. Then the monoliths were placed into a vial containing 12 ml of ConA solution (4 mg/ml) in buffer A. The incubation was carried out at room temperature for 20 hours at continuous shaking. Unreacted epoxy groups were blocked by incubating the cryogels with 14 ml of 0.1 M ethanolamine in buffer A for 2 hours at room temperature using a shaker. Finally, the modified cryogels were placed into the separation tray and washed with 0.1 M acetate buffer pH 6.5 containing 0.5 M NaCl, 1 mM CaCl2, 1 mM MgCl2 (buffer B). ConA cryogels were kept at +4° C in buffer B.

Example 10. Direct quantification of yeast cells by their metabolic activity using ConA-cryogel monolith separation tray

Yeast cells were suspended in 0.95 mM CaCl2, 5.56 mM KCl, 137 mM NaCl, 0.8 mM KH2PO4, 0.41 mM NaHCO3 and 0.01 mM TRIS pH 7.4 (buffer D). Different amounts of yeast cells were applied to the wells packed with ConA-cryogel monoliths (0.5 ml) equilibrated with buffer D in the wells of a separation tray prepared according to Example 9. After 10 min incubation the wells were washed with 1.5 ml of buffer D to remove unbound cells. Then the wells were incubated for 3 hours with 0.5 ml glucose (50 mM) solution in buffer D containing neutral red (0.2 mg/ml). Then the reaction liquid was displaced with 1 ml of deionized water and analyzed by measuring absorbance at 528 nm (Table 3). Absorbance at 528 nm of a solution contain-

ing neutral red changes linearly with pH. Yeast cell metabolism involves production of protons and results in decrease of pH of the medium. Change in absorbance at 528 nm in the medium containing yeast cells, glucose and neutral red allows calculation of change in metabolic activity which is proportional to the number of immobilized viable cells.

Table 3. Change in absorbance caused when different amounts of yeast cells were bound to randomly selected wells packed with ConA-monoliths. Amount of cells is expressed in units of turbidity measured as a difference adsorbance at 600 nm between applied and eluted).

Relative cell number	0	0.031	0.102	0.232	0.339	0.418
ΔA528	0.744	1.130	1.623	2.243	2.804	2.920

Example 11. A competitive binding assay of carbohydrates and glycoenzyme using cona-cryogel separation trays

The binding capacity of the immobilized ConA was estimated by applying different amounts of pure glycoenzyme, horse radish peroxidase, to ConA-cryogel monoliths of a separation tray prepared according to Example 9 equilibrated with 0.1 M TRIS buffer pH 7.0 containing 1 mM MgCl2 and 1 mM CaCl2 (buffer E). After the adsorption stage the monoliths were washed with 2 ml of buffer E containing 1 M NaCl to remove unspecifically bound peroxidase. The amount of bound protein was determined by calculating the difference between the amount of applied and unbound protein (Table 4).

Table 4. Binding of horse-radish peroxidase. to randomly selected wells packed with Con-A cryogel monoliths .

Applied peroxidase, µg	0	12	25	37	50	75	123	196	294	392
Bound peroxidase, µg	0	6	12	13	14	19	25	31	32	31

A competitive binding assay (Table 5) was performed as follows: To 0.5 ml of a glucose containing sample 15 µl of peroxidase solution containing 6 µg of enzyme was added. Samples containing different amounts of glucose and 6 µg of peroxidase were applied to randomly selected wells packed with ConA-cryogel monoliths. After 5 min incubation the wells were washed with buffer C containing 1 M NaCl. Then 0.5 ml of substrate solution (o-phenylenediamine dihydrochloride) was added and left within the wells for 10 min. The reaction mixture was displaced by adding 0.5 ml of buffer E and analyzed by measuring absorbance at 520 nm.

Table 5 Competitive binding assay for glucose. Relative inhibition value (residual peroxidase activity) is given as a function of glucose concentration.

Peroxidase activity, %	6	8	16	32	45	50	60	70	80	100
Glucose concentration, mM	100	90	72	60	50	41	33	23	19	19